# EXHIBIT BV

Supplementary report regarding *CALM2* variant NM_001743.6(*CALM2*): c.340G>A p.Gly114Arg

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This supplementary report is prepared in response to new information regarding a variant in the *CALM3* gene, p.Gly114Trp. We refer to a paper published in June 2019 by Crotti et al (electronically published ahead of print; https://doi.org/10.1093/eurheartj/ehz311 ; hereafter "Crotti et al") and to a letter from Prof Peter Schwartz. The paper of Crotti et al describes data collated from 74 subjects having a "calmodulinopathy"; ie a cardiac condition believed to be related to variants in the calmodulin genes. It is an international report collated from more than 22 centres, and includes previously reported small series and case reports. It is the largest report to date of this very rare condition. The lead authors were Drs Crotti, Ackerman and Schwartz.

1. **Background.** In our original submission and in evidence given to the enquiry, we discussed the CALM2 variant p.Gly114Arg. This variant was found in Kathleen, Sarah and Laura Folbigg, but not in Caleb and Patrick Folbigg.

1.1 Based on the evidence available at the time, we assessed the variant as a Variant of Uncertain Significance. Our view was that the available evidence made it unlikely that this variant was relevant to the deaths of Sarah and Laura Folbigg.

1.2 Within the ACMG framework for assessing variant pathogenicity, we applied the following criteria: PM2 (the variant is absent from controls); PP3 (computational evidence supports pathogenicity); BS2 (variant observed in a healthy adult in a condition in which it would be expected that a person with a pathogenic variant would show features).

1.3 We did not apply the criterion PP2 (Missense variant in a gene that has a low rate of benign missense variation) because the data for this gene from the population database gnomAD indicate that this variant does not meet the usual threshold for applying this criterion. During the hearing, Professor Vinuesa argued that the region where this variant has occurred does have a low rate of benign variation.

On May 22, 2019 (subsequent to the substantive hearings into evidence relevant to genetics, cardiology and neurology that occurred in the week commencing 15 April 2019) the publication "*Wiel et al. (2019). MetaDome: Pathogenicity analysis of genetic variants. Human Mutation 10.1002/humu.23798. PMID: 31116477"* became available in advance online form in the medical journal *Human Mutation*. The methodology proposed in this article allows the tolerance to missense substitution of amino acid 114 in CALM2 to be quantified. Results indicate that the tolerance score (dn/ds) at this residue is 0.05, which is highly intolerant to missense substitution. Therefore, with the availability of quantitative evidence regarding Prof Vinuesa's proposition, we agree that criterion PP2 can be used.

1.4 Adding the criterion PP2 does not change the classification of the variant as a Variant of Uncertain Significance.

1.5 It is important to understand that almost all of the previously reported disease-associated variants in the three *CALM* genes, *CALM1, CALM2* and *CALM3* (all three of which code for an identical protein) involve amino acids located within two specific regions ('domains') of the protein. These are called EF-hand III and EF-hand IV. Only three amino acids outside those two domains had been associated with disease prior to the new report from Crotti et al. Those three are in a different part of the protein from the location of the Gly114 amino acid. In other words, there had never been a report of a change in the protein in the vicinity of Gly114 that was disease-associated.

1.6 We applied the criterion BS2 on the grounds that a variant that resulted in two deaths at ages 10 and 20 months must, by definition, be a cause of very severe disease. The fact that Kathleen Folbigg is alive and has no clear evidence of any cardiac illness is inconsistent with her having such a condition. We note that Professor Skinner's opinion was that that the documented fainting events for which we have available medical records were not typical for cardiac events, and that the resting 12 lead ECGs were normal, with normal QT intervals, and he noted that an exercise test had been reported as normal previously. Subsequent to that, Kathleen

Folbigg was seen and assessed by Dr Hari Raju. He took a clinical history wherein he reviewed in particular the reported syncopal events, and performed an exercise test himself, and reviewed a 24 hour Holter recording. He concluded that Kathleen had no phenotypic evidence of either cardiomyopathy or a primary arrhythmia syndrome.

1.7 Where there is information about the parents of a person affected by a *CALM*-related condition, it is almost always found not to have been inherited from a healthy parent. Crotti et al state that where parents have been tested, the variant is almost always *de novo* (neither parent has the change, i.e. it is new in the child) *or* is inherited from a parent with gonadal mosaicism (i.e. where the change is present in ovaries or testes but not in the rest of the body). For 27 of 29 cases where the phenotype was long QT, the variant was *de novo*, and for the other two there was gonadal mosaicism. For 7 of 9 with CPVT the variant arose *de novo*. Thus, it is very unusual for a pathogenic variant in these genes to be inherited from a parent, particularly an unaffected parent.

1.8 The available information about conditions associated with the *CALM* genes indicates that variants affecting amino acids outside the EF-hand domains lead to generally less severe medical conditions than variants affecting EF-hand amino acids – although these are still life-threatening, with deaths reported in 14% of known individuals with these 'less severe' conditions. The main specific condition is catecholaminergic polymorphic ventricular tachycardia (CPVT). Idiopathic ventricular fibrillation (IVF) is also reported. However, for all types, 81% of cardiac events were the result of adrenergic stimuli; 62% were associated with exercise. This is relevant because of the history that Sarah and Caleb were described as having been found deceased during a sleep period, which is not consistent with a diagnosis of CPVT. To our knowledge, variants in calmodulin have still not been reported as a cause of sudden infant death syndrome, being death of an infant during sleep. Their young age is also not typical for calmodulinopathies. The median age of sudden cardiac death in the registry was 5.7 years, and most of the youngest deaths were associated with the long QT phenotype - which was never seen in a child of a parent carrying a non-mosaic variant.

1.9 The youngest age of death that we can find documented in the medical literature for a patient with a non-EF-hand *CALM* variant is 4 years.

1.10 We note that in their report, Professors Vinuesa and Cook considered co-segregation of the variant with phenotype as a possible additional supporting criterion. The principle of co-segregation with phenotype is that if there are multiple individuals in a family affected by a rare condition, it is expected that if there is a disease causing variant, it will be identified in all of the affected individuals. It also generally will not be present in unaffected individuals, except for conditions in which non-penetrance is common. An essential prerequisite for considering co-segregation as a form of evidence is that it should be possible to classify family members as affected or unaffected before any genetic data are available. In this family, the phenotype is death at a very young age during a sleep period. The affected individuals are thus the four children. Possible genetic mechanisms which could lead to application of the co-segregation criterion would be a dominant condition with gonadal mosaicism in one parent, or an autosomal recessive condition with all four children inheriting a faulty copy of a gene from each parent. Since there was no reason prior to the genetic results to consider Sarah and Laura as likely to have had a different genetic cause of death from Caleb and Patrick, if co-segregation were being considered as a criterion, identification of a variant in only two of the four children would represent strong evidence against pathogenicity of that variant. Certainly this could not be considered evidence in favour of pathogenicity, and this criterion cannot be applied as supporting pathogenicity of the CALM2 variant. Any discussion of the CALM2 variant as a possible cause of death for Sarah and Laura must necessarily assume that there are at least two different causes of death for the children – one for Sarah and Laura, and one or more for Caleb and Patrick.

1.11 We also note that Professors Vinuesa and Cook applied PP4 – "Patient's phenotype or family history is highly specific for a disease with a single genetic aetiology". As discussed previously, sudden death is not a condition with a single genetic cause and thus PP4 is not relevant in assessing whether this variant may be pathogenic.

2. New information and its significance. Crotti et al report a family with a variant in *CALM3,* p.Gly114Trp.

2.1 Although this involves a different gene, the information is relevant, because the three *CALM* genes encode an identical protein.

2.2 The information that it is possible for a change affecting this amino acid to be disease causing has a significant impact on the likelihood that the CALM2 Gly114Arg variant could be pathogenic. It is a different amino acid substitution, but it is in the same position as one which we now know can be disease causing.

2.3 There is limited clinical information relating to the Gly114Trp variant in Crotti et al. However, in Professor Schwartz's letter there is additional information. Specifically, the variant was found in a brother and sister. The boy died suddenly aged 4 (no further information is available). The girl suffered a cardiac arrest while playing, aged 5. The children's mother was found to be mosaic for the variant. This means that there was evidence that she had some cells (presumably including some in her ovaries) which had the variant, whereas others did not. Having a significant number of cells in her heart without the variant would be expected to be protective.

2.4 Professor Schwartz suggests that studies should be undertaken to search for evidence of mosaicism in Kathleen Folbigg. The reason for this suggestion is not stated but presumably it is because of the information provided above, that it would be very unusual to have a disease causing variant responsible for deaths at such young ages also present in a healthy person in her 50s; and that therefore mosaicism may be the explanation. Professor Schwartz was evidently unaware that Kathleen had undergone two exercise tests, with the second test being done explicitly to consider QT measurements during recovery after exercise and features of CPVT during it. These clinical tests were reviewed by two specialists in inherited cardiac conditions, and they came to the same conclusion; that these were within normal limits. There were 2 or 3 isolated monomorphic ventricular ectopics during the exercise test, which while common in the healthy population of over 50 year olds, could possibly be a very weak sign of concealed CPVT, even though there was no ventricular tachycardia, and no polymorphic or bidirectional beats more typical for CPVT. These results and the fact that she is alive and has never had a cardiac arrest is strongly against this hypothesis.

2.4.1 Regarding the genetic evidence specifically, in 82 reads (reflecting 82 separate strands of DNA) in the whole genome sequencing data from Kathleen Folbigg's sample, there is a 40:42 split between the normal and the variant base pair. By way of context, in people who are truly heterozygous for a variant (carry a copy of it in all cells), there can be a range of proportions seen in sequencing data purely by chance. As an example, in Laura and Sarah Folbigg's samples we see 41% C/59% T and 54% C/46% T respectively. We sometimes see as few as 20% of the reads representing one of the two copies of a stretch of DNA, even though in fact they are present in equal proportions. In cases with mosaicism we always see a clear reduction in the representation of the variant copy.

2.4.2 Nonetheless, it is *theoretically* possible to have mosaicism in one tissue (heart) which is not reflected at all in another (blood), which could explain the absence or greatly attenuated cardiac phenotype in Kathleen. Our opinion is that based on the available genetic data, this is very unlikely, and that testing other tissues is thus unlikely to be of value.

2.5 Adding this new information to the previously available information relevant to the p.Gly114Arg variant means that an additional moderately strong criterion should be applied, PM5: Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.

2.6 The addition of functional studies has been suggested. At present, there are no functional studies for this protein which are validated to clinical standards. *In vitro* studies of protein function are notorious for both false positives and false negatives. Thus, a positive *in vitro* study would not prove that the variant is pathogenic, and a negative study would not prove that it is benign.

2.7 If the clinical information described above is *not* taken into account, the addition of PM5 to the previously applied criteria, PM2, PP2 and PP3 would mean that the variant would be classified as Likely Pathogenic (class IV) with a 10% residual likelihood that this assessment of the level of pathogenicity is incorrect.

2.8 However, the clinical and genetic information are conflicting, and it is our opinion that it is essential to consider the clinical information in interpreting the significance of the variant.

## 3. Possible interpretations of the available information.

3.1 *The variant could be pathogenic and could be the sole cause of the deaths of Sarah and Laura.*
This would require a clinical scenario which is out of the range that has been reported previously in relation to the *CALM* genes, with two deaths at a very young age in children who have inherited the variant from a parent who is alive and well in her 50s. The variant is of a type that (if pathogenic) would not usually be expected to cause death at such a young age; would not be expected to cause death in sleep; and would almost always be expected to have arisen *de novo* or have been inherited from a parent with mosaicism for the variant. For all these reasons, this clinical scenario would be exceptional. However, this is a very rare group of conditions and the full range of possible clinical manifestations is not known. Given the new information, this scenario is considered possible.

3.2 *The variant could be pathogenic and related to, but not the sole cause of, the deaths of Sarah and Laura.*
An alternative clinical scenario would be that Sarah and Laura experienced events which may or may not have been normally sufficient to cause their deaths (such as an asphyxial event), but which, through adrenergic stimulation induced a cardiac arrhythmia which otherwise would not have happened, leading to their deaths. This scenario is considered possible.

3.3 *The variant could be pathogenic, but unrelated to the deaths of Sarah and Laura*.
This scenario is considered possible.

3.4 *The variant could be benign.*
There are numerous examples of situations in which one change to an amino acid is pathogenic whereas another, at the same residue, is benign. Even within the *CALM* genes, there is evidence for an example of this; the change of asparagine 138 to lysine has been reported in association with the very severe Long QT phenotype, whereas a change of the same amino acid to a different amino acid, serine, has been reported in a healthy population control. In the case reported by Crotti et al, the glycine at position 114 is changed to tryptophan; in the Folbigg family the change is to arginine, which is chemically very different from tryptophan. Thus, it is possible that the Gly114Arg variant is benign.

## 4. In conclusion,

the information regarding the existence of a different pathogenic variant at Gly114 is relevant to interpretation of the potential clinical significance of the Gly114Arg variant, increasing the likelihood that this variant is pathogenic and that it might be relevant to the deaths of Sarah and Laura. As discussed above, for this to be the sole cause of death for Sarah and Laura, an exceptional clinical scenario would be required – outside the range that has previously been reported in association with variants in this group of genes. It is possible that the variant contributed to, but was not the sole cause of, the deaths of Sarah and Laura. Either of these first two possibilities would require at least two different causes of death for children in the family, because Caleb and Patrick did not inherit the *CALM2* variant.

It is possible that the variant is pathogenic but is unrelated to the cause of death of Sarah and Laura. Lastly, it is possible that the variant is benign and unrelated to their deaths. Because of the conflict between the clinical and genetic evidence, our classification of this variant remains that it is a Variant of Uncertain Significance (Class III). In keeping with this classification, we are uncertain which of the possibilities described above is correct, and do not see a straightforward way of resolving the issue by further genetic or other testing. It may be that only further reports of the same variant (either in healthy individuals or in a setting in which it is clear that it is pathogenic) will resolve the issue of pathogenicity. Even then, genetic information alone will not determine whether there may have been other contributing factors relevant to the deaths.